Multi-system neuroprosthetic training improves bladder function after severe spinal cord injury

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Abstract: BACKGROUND: Severe spinal cord injury (SCI) leads to neurogenic bladder dysfunction. We recently developeda multi-system neuroprosthetic training program (MSNT) that promotes plastic changes capable of restoring refinedlocomotion in rats with severe SCI. We investigated whether MSNTinfluences the formation of posttraumatic bladder dysfunction. MATERIALS AND METHODS: Adult rats were randomly assigned to a SCI (n=8) and to a control intact (n=4) group. SCI consisted of two opposite lateral hemisections (T7, T11), thusinterrupting all direct supraspinal input. After SCI, 4 rats were subjected to MSNT, 4 rats were non-trained. After 8 weekswe performed urodynamics and evaluated kidney function (creatinine, cystatin C). Bladder investigation included morphological, histological and immunohistochemical evaluations. RESULTS: Bladder capacity increased 3-fold in trained and 7-fold in non-trained compared to intact animals. During filling,we found 2.7 ± 1.1 non-voiding contractions (NVC) in trained, compared to 12.6 ± 5.2 in non-trained rats. Bladder morphology was similar in trained and intact rats, non-trained ratsexhibited detrusor hypertrophycharacterized by increased detrusor thickness and decreased connective tissue to smooth muscle ratio. The general nerve density, labeled with PGP9.5, was significantly increased in trained and significantly decreased in non-trained rats. The relative proportion of NF200-postive afferent nerves was significantly lower in trained compared to intact and non-trained rats. NPY-positive fibers showed a significantly lower density in non-trained rats. CONCLUSIONS: MSNT effectively counteracts the formation of neurogenic bladder dysfunction after severe SCI and might contribute to preserving bladder function and preventing long-term complications in patients with severe SCI.

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MULTI-SYSTEM NEUROPROSTHETIC TRAINING IMPROVES BLADDER FUNCTION AFTER SEVERE SPINAL CORD INJURY

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Runninghead neuroprosthetic training improves bladder function

Key words bladder function, bladder innervation, neuro**genic** bladder, rehabilitation, spinal cord injury

ABSTRACT

Background: Severe spinal cord injury (SCI) leads to neuro**genic** bladder dysfunction. We recently developeda multi-system neuroprosthetic training program (MSNT) that promotes plastic changes capable of restoring refinedlocomotion in rats with severe SCI. We investigated whether MSNTinfluences the formation of posttraumatic bladder dysfunction.

Materials and Methods: Adult rats were randomly assigned to a SCI (n=8) and to a control intact (n=4) group. SCI consisted of two opposite lateral hemisections (T7, T11), thusinterrupting all direct supraspinal input. After SCI, 4 rats were subjected to MSNT, 4 rats were non-trained. After 8 weekswe performed urodynamics and evaluated kidney function (creatinine, cystatin C). Bladder investigation included morphological, histological and immunohistochemical evaluations.

Results: Bladder capacity increased 3-fold in trained and 7-fold in non-trained compared to intact animals. During filling,we found 2.7 ± 1.1 non-voiding contractions (NVC) in trained, compared to 12.6 ± 5.2 in non-trained rats. Bladder morphology was similar in trained and intact rats, non-trained ratsexhibited detrusor hypertrophycharacterized by increased detrusor thickness and decreased connective tissue to smooth muscle ratio. The general nerve density, labeled with PGP9.5, was significantly increased in trained and significantly decreased in non-trained rats. The relative proportion of NF200-postive afferent nerves was significantly lower in trained compared to intact and non-trained rats. NPY-positive fibers showed a significantly lower density in non-trained rats.

Conclusions:MSNT effectively counteracts the formation of neurogenic bladder dysfunction after severe SCI and might contribute to preserving bladder function and preventing long-term complications in patients with severe SCI.

1. INTRODUCTION

Normal urinary bladder function comprises storage and emptying of urine in a coordinated, controlled fashion. Bladder emptying involves a complex synergy between the pontine micturition center, the sympathetic and parasympathetic nervous system and motoneurons in the spinal cord[1]. Spinal cord injury (SCI) severely impacts bladder function, and leads to neurogenic bladder dysfunction. SCI initially causes acontractile detrusor associated with urinary retention, which induces bladder overdistension. This early response is gradually superseded by the development of an automatic spinal micturition reflex with involuntary bladder contractions[2]. This neurogenic detrusor overactivity leads to simultaneous contractions of bladder and sphincter, referred to as detrusor sphincter dyssynergia (DSD). Increased detrusor pressure and urinary retention result in alterations in bladder size and tissue composition [1, 3]. Further deterioration of bladder function and frequent infections predispose renal damage and renal failure. Therefore, preventing neurogenic bladder dysfunction is essential to decrease morbidity and improve the quality of life of individuals with severe SCI[4].

A recent case study conducted in a young male suffering chronic complete paraplegia reported that motor training enabled by electrical spinal cord stimulation not only restored the capacity for full weight-bearing standing, but also mediated the unexpected recovery of voluntary voiding[5]. Presumably, use-dependent mechanisms promoted the remodeling of fibers spared by the lesion[6]. Indeed, we recently showed that MSNT, an electrochemically enabled and robot-assisted rehabilitation[7], triggered a massive reorganization of descending and intraspinal pathways in rats with severe SCI[8]. This extensive plasticity restored voluntary control over refined locomotor movements in otherwise paralyzed rats.

Here, we characterized the development of neurogenic bladder dysfunction and investigated whether MSNT is capable of improving bladder function in rats with severe SCI.

2. MATERIALS AND METHODS

2.1. Study design

Twelve adult female Lewis rats (220 g) were randomized into three groups: a trained SCI group (n=4), a non-trained SCI group (n=4) and an intact control group (n=4).The effect of MSNT on bladder function was assessed 8 weeks post-SCI by urodynamics. The rats were sacrificed one week later, and systemic blood parameters and histological evaluations of the bladder wall and its innervation were performed.

2.2. Surgical procedures and post-surgical care

All procedures followed the Care and Use of Laboratory Animals, approved by the local Veterinary Office, and have been described in details previously[8]. Briefly, under general anesthesia, we implanted chronic stimulating electrodes over the dorsal aspects (epidurally) of spinal segments L2 and S1. Bipolar intramuscular EMG electrodes were inserted bilaterally in the medial gastrocnemius and tibialis anterior muscles. In a second surgery, mid-thoracic partial laminectomies were performed and the spinal cord was hemisected laterally on opposite side at levels T7 and T10. This lesion was selected for his clinical relevance and heuristic value to investigate mechanisms underlying recover[8]. Completeness of the lesion was verified histologically post-mortem. In all animals the bladders were manually expressed twice daily until sacrifice.

2.3. Electrochemical neuroprosthesis

To encourage locomotion via electrical spinal cord stimulation, rectangular pulses were delivered through L2 and S1 electrodes (40 Hz, 0.2 ms duration, 200-400µA)[9]. Pharmacological modulation was induced by administrating receptor agonists for 5-$HT_{2A/C}$ (quipazine, 0.2-0.3 mg/kg, i.p.), 5-$HT_{1A/7}$ (8-OHDPAT, 0.05-0.3 mg/kg, s.c.)[10], and dopamine D1 (SKF-82197, 0.1-0.2 mg/kg, i.p.) 10 minutes prior to locomotor training.

2.4. Multi-system neuroprosthetic training

MSNT started seven days post injury and was performed six days per week for 25min per session. The rats were subjected to treadmill-based training with vertical support (Robomedica, USA) in order to optimize functionality of lumbosacral circuits. At the end of each session, we positioned the rats in the robotic postural interface and encouraged them to walk bipedallyoverground towards a target located in front of them. As the rats progressively regained voluntary locomotion, we gradually increased the relative duration of overground training.

Behavioral recording procedures and data analysis have been described in detail previously[8]. To quantify recovery, we adapted the 6-min walk test to bipedally walking rats. We calculated the distance covered in 3 minutes during robot-supported bipedal locomotion overground.

2.5. Urodynamic study

Under isofluraneanaesthesia (2.5%), a 26GA catheterwas inserted transurethrally. The bladder was emptied and the tubing connected to the pressure transducer (AD Instruments, Germany). Prior to recording, the isoflurane level was reduced (1%) to minimize effects on bladder function. Saline at room temperature was infused via an infusion pump (10ml/h) and intravesical pressure was recorded. Maximal bladder capacity (MBC) was determined as the volume at which leakage occurred. Detrusor contractions of>4cmH_2O not associated with leakage, were referred to as non-voiding contractions (NVC)[11].

2.6. Morphology and Histology

Nine weeks post-surgery the rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (40mg/kg). Blood samples were withdrawn from the sublingual vein, to

measure serum creatinine and cystatine C. Bladders were removedand fixed with 4% formaldehyde and later paraffin-embedded. Bladder sections (5µm) were stained by routine hematoxylin-eosin and Masson's trichrome.Histological images were taken (Leica, Germany) and quantitative measurements were performed using Image J software (NIH Image, Bethesda, MD) to determine detrusor thickness and connective tissue to smooth muscle ratio (Co:SM ratio).

2.7. Immunohistochemistry and Histomorphometry

Bladder sections were processed for specific neural markers. Sections were incubated overnight in the following primary antibodies: rabbit anti-protein gene product 9.5 (PGP9.5, 1:500, Millipore, USA), rabbit polyclonal to Neuropeptide Y (NPY, 800, Abcam, UK), rabbit anti-neurofilament 200 (NF200, 1:300, Sigma-Aldrich,, USA), rabbit polyclonal to tyrosine hydroxylase (TH, 1:500, Abcam) and rabbit polyclonal to vesicular acetylcholine transporter C-terminal (VAChT, 1:200, Abcam). Fluorescein anti-rabbit IgG (1:1000, Vector Laboratories, Burlingame, CA, USA) was used as secondary antibody. Analysis was carried out using a Leica DM6000 fluorescence microscope. To quantify bladder innervation the number of stained fibers per high-power field (200x) was counted. For each staining, a total of 8 separate HPF were analysed blindly for each bladder, and the mean value was used for statistics.

2.8. Statistical analysis

All data were expressed as mean and standard error of the mean (SPSS 18.0, SPSS, USA). To assess the difference between the three groups one-way analysis of variance (ANOVA) with a Bonferroni post-hoc analysis was performed. Statistical significance was set at $p < 0.05$.

3 Results

3.1. Multi-system neuroprosthetic training

The staggered hemisections (**Figure 1C**) induced complete and permanent hindlimb paralysis, both in trained and non-trained rats. To enable locomotion, we applied an electrochemical neuroprosthesis(**Figure 1B**). Without training, electrochemical stimulations promoted hindlimb movements on a treadmill, but non-trained rats failed to initiate and sustain voluntary locomotion overground (0.6 ± 0.27m, $p<0.001$) **(Figure 1E)**.In contrast, trained rats regained the capacity to produce full weight bearing overground locomotion for extended periods of time in the presence of electrochemical stimulations (**Figure 1F**). Trained animals covered distances as long as 21m in 3min (10.6 ± 1.26m, $p<0.001$) **(Figure 1G**), which corresponds to continuous locomotion at a sustained pace in intact rats.

3.2. Urodynamic study

In intact rats, bladder pressure increased slowly during bladder filling until a sharp pressure increase initiated the voiding contraction **(Figure 2A)**. In contrast, rats with severe chronic SCI exhibited repeated NVC during filling. Non-trained rats showed strong detrusor overactivity, starting at 55% of MBC **(Figure 2B, Table 1)**. Trained rats showed significantly lesser NVC and a delayed onset of NVC (77% of MBC). MBC was significantly ($p<0.005$) lower in trained compared to non-trained rats, although both groups showed increased MBC **(Figure 2B, Table 1)**. Leak point pressure (LPP) was in the normal range in all groups **(Figure 2D, Table 1)**.

3.3. Morphology and Histology

Normal serum creatinine and cystatin C levels **(Table 2)** and macroscopically normal upper urinary tracts exclused the occurrence of renal damage in rats with SCI. The bladders were considerably enlarged in non-trained rats. Cross-sectional detrusor thickness analysis

highlighted a significant detrusor thickening ($p<0.005$) in non-trained rats compared to intact and trained rats. Significant decrease in Co:SM ratio ($p<0.01$) confirmed detrusor hypertrophy in non-trained rats **(Figure 3, Table 2).**

3.4. Immunohistochemistry

To evaluate bladder innervation, we quantified the density of nerves in the bladder wall using different neurochemical markers **(Figure 4, Table 3)**. Immunoactivity for PGP 9.5 as a general nerve marker was significantly increased ($p<0.001$) in trained rats and significantly ($p<0.001$) decreased in non-trained rats. Evaluation of afferent fiber innervation with NF200 antibodies revealed a significantly reduced A-fibers density in non-trained rats **(Figure 4E)**. To determine the afferent A-fiber fraction, the NF200:PGP ratio was calculated. The NF200:PGP ratio was significantly lower in trained compared to intact and non-trained rats. Fibers labeled with NPY showed a significantly lower density in non-trained rats **(Figure 4F)**. Analysis of TH- and VAChT-positive efferent fibers revealed no significant difference between the groups, although non-trained rats showed a trend for low TH density compared to the other groups.

4. Discussion

Our combined functional, morphological, and immunohistochemical analyses confirm the dramatic impact of a severe SCI on bladder function in rats, and demonstrate the unexpected capacity of MSNT to prevent the formation of neuro**genic** bladder dysfunction. These new findings open promising therapeutic avenues to prevent long-term bladder related complications in patients with SCI.

4.1. Formation of neurogenic bladder dysfunction

Our SCI model completely interrupts the spino-bulbo-spinal micturition reflex pathway, abolishes the supraspinal control of bladder and sphincter functions, and thus leads to DSD[12]. The resulting functional outlet obstruction causes bladder distension and detrusor hypertrophy[13]. These changes elicit profound, long-lasting alterations in the pattern of autonomic bladder innervation, which leads to a pathological increase in neural excitability [2, 14]. In agreement with these well-established changes, we found a 7-fold increase in bladder capacity, substantial detrusor hypertrophy, and significant bladder overactivity in non-trained rats with severe SCI.

In intact rats, supraspinal reflex pathways mediate the micturition reflex through the activation of NF200-positive myelinated Aδ-fiber afferents, which constitute 30% of bladder sensory innervation[15]. A complete SCI induces a shift from afferent C-fibers to Aδ-fiber phenotypes, which leads to a2-fold increase in the proportion of NF200-positive projections in the chronic SCI state[15]. Likewise, we found a substantial increase in the relative proportion of NF200-postive nerves in non-trained rats with severe SCI. Overdistension of the urinary bladder wall causes the release of neurotrophic factors, including nerve growth factors that are likely responsible for the observed plastic changes[15-17]. Together, these findings suggest that the formation of neurogenic bladder dysfunction results from the progressive increase in afferent Aδ-fiber density, which enhances excitability of the bladder wall and likely leads to the detrusor overactivity observed in non-trained rats with severe SCI[15, 18].

4.2. Preventing neurogenic bladder dysfunction with MSNT

We exploited an electrochemical spinal neuroprosthesis and a robotic interface to encourage paralyzed rats to walk towards a target. After a few weeks of training, the animals regained voluntary control of locomotion. More unexpectedly, they also exhibited significant improvements of bladder function. MSNT promoted the maintenance of bladder afferent innervation, which likely accounted for the near-absence of non-voiding contractions in

trained rats. Along the same lines, Harkema *et al.* demonstrated that after several months of stand training enabled by electrical spinal cord stimulation, a chronically paraplegic man not only regained voluntary leg movements, but also recovered voluntary bladder and bowel control despite the absence of specific training procedures for these functions[5]. This recovery was interpreted as a result of the general influence of use-dependent mechanisms on the plastic reorganization of descending pathways and local spinal circuits[6].Indeed, we showed that MSNT mediates motor recovery through the massive reorganization of supraspinal and intraspinal axonal systems in rats[8].However, at this stage, the mechanisms that prevent the formation of neurogenic bladder dysfunction remain unclear. It has been shown, that the activation of 5-HT receptors[19], especially 5-HT_{1A} [20] may modulate the pathways involved in the control of micturition. However, daily recruitment of $5HT_{1A}$ receptors is unlikely to account for improvement of lower unrinary tract function in trained rats since these receptors primarely have bladder excitatory effects and lead to prologed urethral opening periods during micturition[20]. Accordingly, we expect that $5HT_{1A}$-mediated effects would be opposite to those observed in trained rats. A possible explanation might relate to the restored daily activity of locomotor-associated lumbosacral circuits in the early posttraumatic period. This initial post-SCI phase is characterized by acontractile detrusor [7], which triggered a series of profound changes in bladder physiology and in afferent bladder innervation in non-trained rats. Robust locomotor-related activity in denervated spinal networks combined with continuous electrical stimulation of lumbosacral segments might suppress sympathetic preganglionic neuron activation in the thoraco-lumbar cord and decrease bladder outlet resistance and limiting detrusor hypertrophy. Furthermore, we can hypothesize that motor recovery mediated by MSNT positively influences somatic innervation of the external urethral sphincter and the pelvic floor, leading to a decrease in bladder outlet resistance.

4.3. Limitations/Weakness

The studied rats were part of a comprehensive investigation on the effect of MSNT on the recovery of motor functions[8]. Consequently, the primary objectives of the study limited the range of potential techniques and interventions for the present investigations. For example, transurethral cystometries were used to limit risks of complications associated with more invasive techniques such as intravesical catheter implantation and measurements in the metabolic cage. Further limitations were the small number of rats investigated and the focus on one single endpoint.

5. Conclusion

These results show that MSNT effectively counteracts the formation of neurogenic bladder dysfunction, and improves bladder function in rats with severe SCI. MSNT limited detrusor hypertrophy, reduced detrusor overactivity, and maintained the bladder innervation pattern globally unaffected. These beneficial effects of MSNT on bladder function might contribute to reducing the risk for upper urinary tract deterioration and preventing long-term complications in patients with SCI.

Conflict of interest

The authors have no financial or personal relations that inappropriately influence the results of the current work.

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Figure Legends

Table 1: Urodynamic parameters

Table 2: Renal function and bladder morphology

Table 3: Immunolabeling of specific nerve fibers in the bladder wall

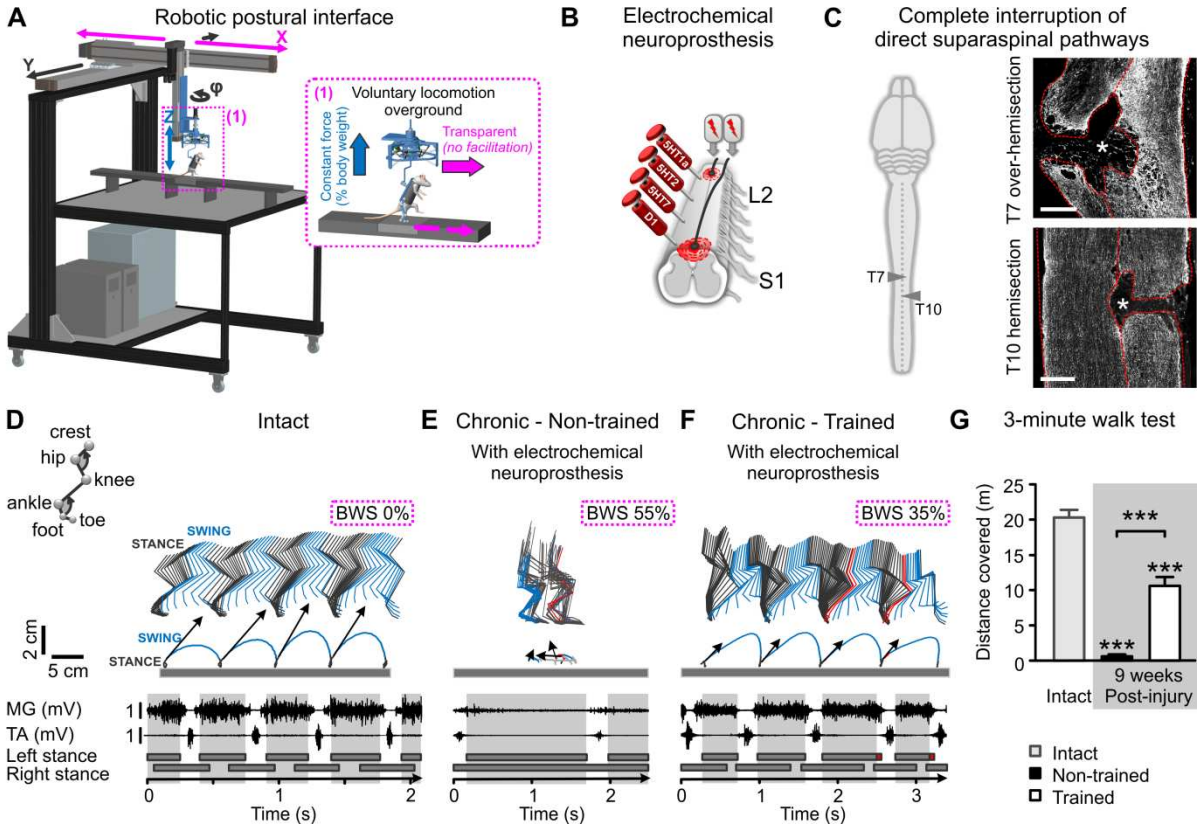
Figure 1: (A) Rats were positioned bipedallyoverground using a robotic postural interface providing vertical and lateral support, but no facilitation in the forward direction. (B) An electrochemical neuroprosthesis was used to enable locomotion after a SCI that completely interrupted all direct supraspinal pathways. (C) Epifluorescent images showing the T7 lateral over-hemisection and T10 lateral hemisection. Scale bar, 500µm. (D)-(F) A representative locomotor trial is shown before the injury (D), and 9 weeks post-injury for a non-trained (E) and a trained (F)rat. For each panel, a stick diagram decomposition of hindlimb motion is shown together with color-coded trajectories of hindlimb endpoints, and the amount of body weight support (BWS). Vectors represent the direction and intensity of the hindlimb endpoint velocity at swing onset. The corresponding sequences of raw EMG activity of an extensor (MG, medial gastrocnemius) and a flexor (TA, tibialis anterior) muscle are displayed below. Grey and red bars indicate the duration of stance and drag phases, respectively. (G) Recovery was measured using the 3-minute walk test that reports the walking distance (m) covered in 3 minutes at the different time points. (***$p<0.001$; Error bars, SEM).

Figure 2: (A) Bladder function was significantly altered following SCI, which was prevented by MSNT as demonstrated by representative bladder filling curves. (B) The number of NVC was significantly higher in non-trained rats. (C) Compared to normal values functional bladder capacity was 3 times increased in trained and 7 times increased in non-trained rats. (D) LPP was significantly decreased in trained rats compared to normal values. (*$p<0.05$)

Figure 3: (A) Detrusor thickness and connective tissue to smooth muscle ratio 9 weeks after complete SCI. HE staining of the bladder wall, showing the increased thickness of the detrusor in the non-trained group. (B) Quantification of detrusor thickness revealed a significant increase in non-trained rats. No difference was detected between trained and intact rats. (C) Co:SM ratio significantly decreased in non-trained rats, indicating detrusor hypertrophy, whereas the ratio did not differ in trained rats compared to intact rats. (*$p<0.05$)

Figure 4: (A-C) Immunolabeling for NPY-positive nerves in the bladder wall revealed decreased density of labeled nerves in non-trained rats compared to the trained and control animals. (D-F) Quantitative analysis showing significantly decreased general nerve staining, afferent and efferent nerve density in non-trained rats compared to trained and control rats. (*$p<0.05$)

Figure 1


A
Robotic postural interface
X
Y
Z
φ
(1)
(1) Voluntary locomotion overground
Constant force (% body weight)
Transparent (no facilitation)
B
Electrochemical neuroprosthesis
5HT1a
5HT2
5HT7
D1
L2
S1
C
Complete interruption of direct supraspinal pathways
T7
T10
T7 over-hemisection
T10 hemisection
D
Intact
crest
hip
knee
ankle
foot
toe
BWS 0%
STANCE
SWING
2 cm
5 cm
MG (mV)
TA (mV)
Left stance
Right stance
Time (s)
E
Chronic - Non-trained
With electrochemical neuroprosthesis
BWS 55%
Time (s)
F
Chronic - Trained
With electrochemical neuroprosthesis
BWS 35%
Time (s)
G
3-minute walk test
Distance covered (m)
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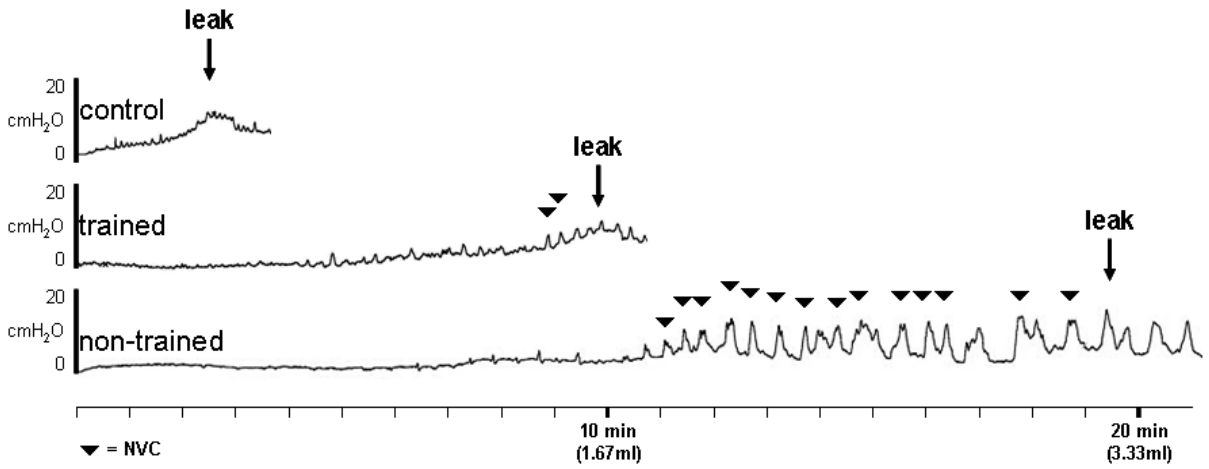
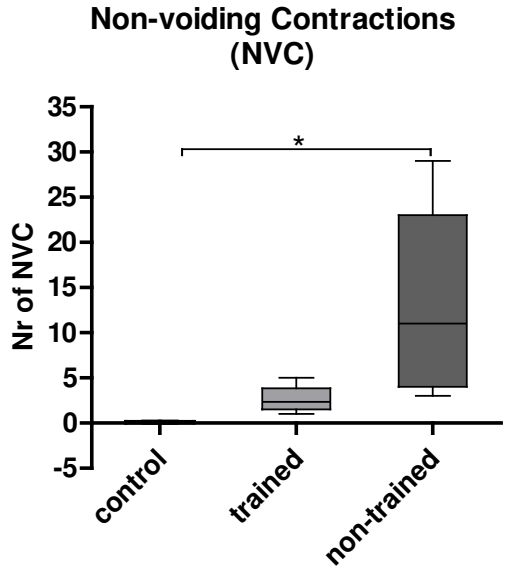
Intact
9 weeks Post-injury
Intact
Non-trained
Trained

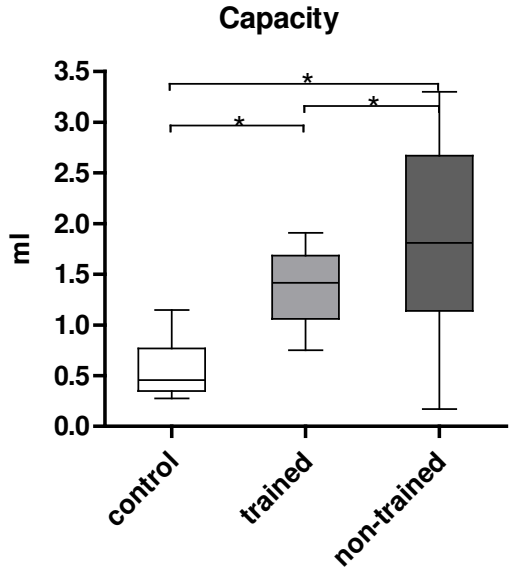
Figure 2



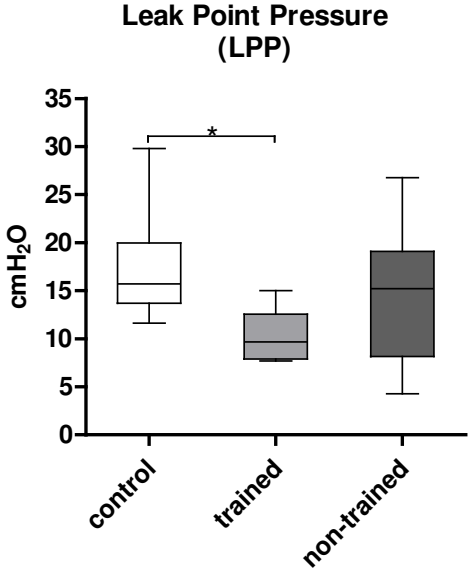
A



B

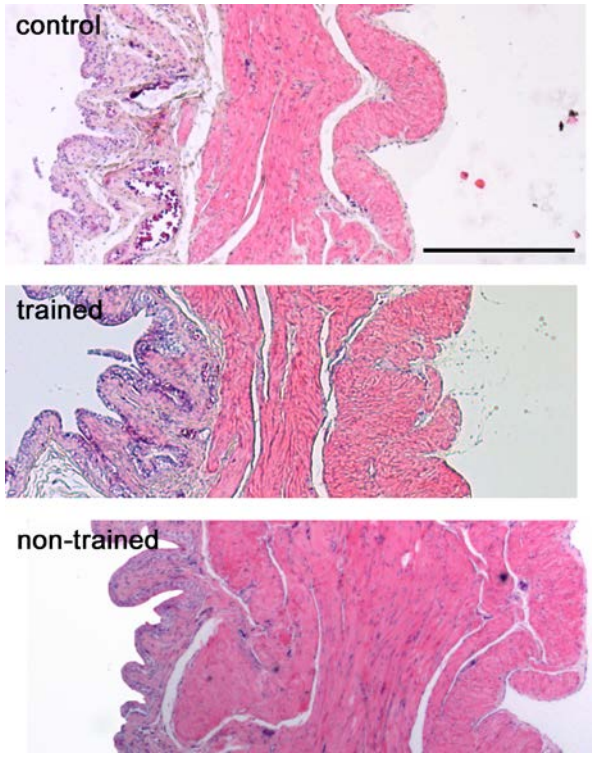


C

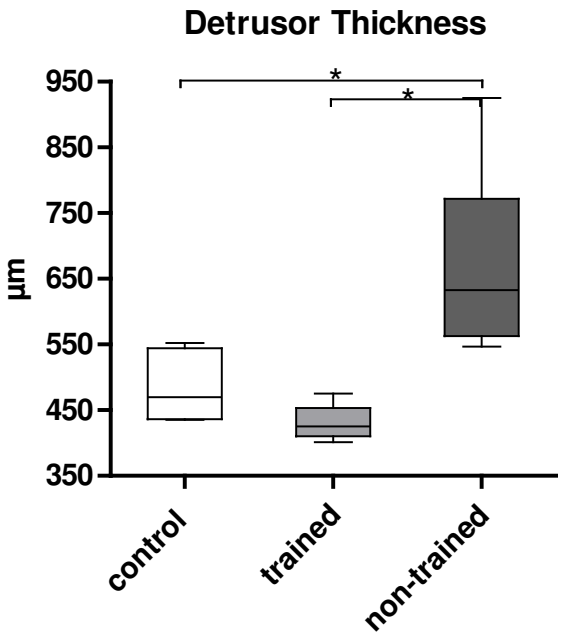


D

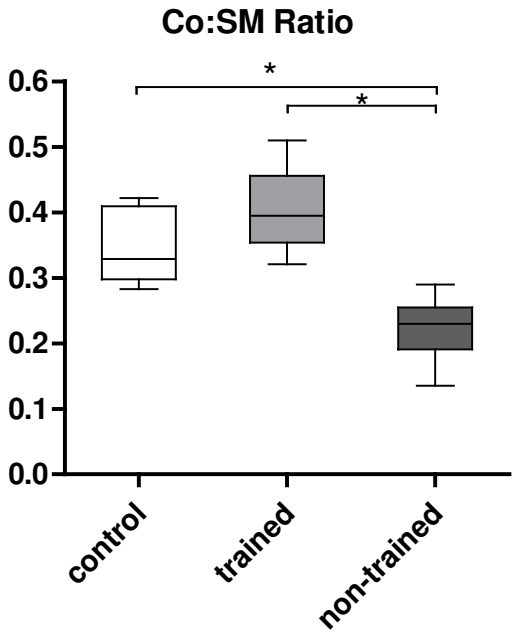
Figure 3


control
trained
non-trained

A


Detrusor Thickness
µm
950
850
750
650
550
450
350
*
*
control
trained
non-trained

B


Co:SM Ratio
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
control
trained
non-trained

C

Figure 4

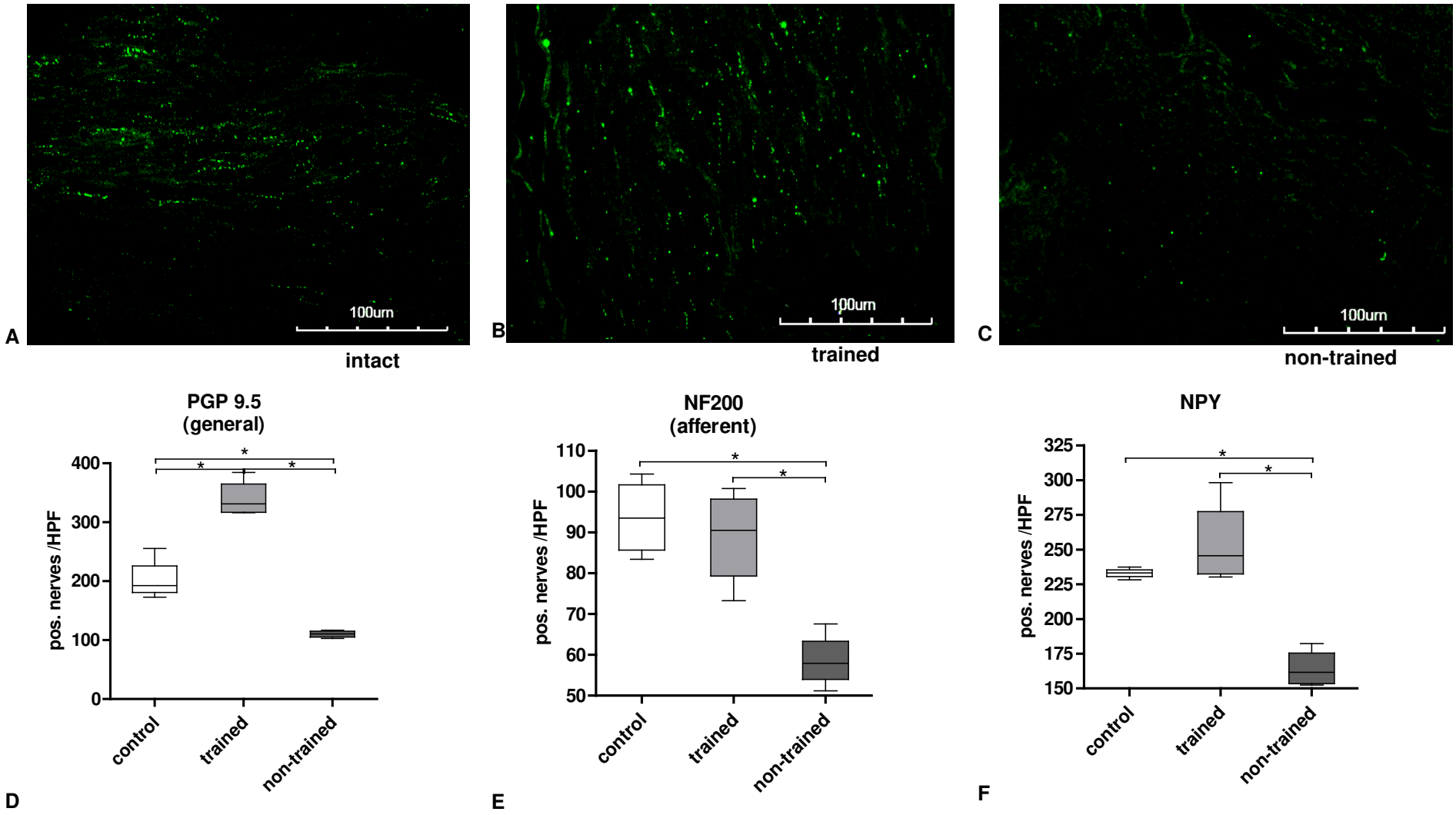

A
100um
intact
B
100um
trained
C
100um
non-trained
PGP 9.5
(general)
pos. nerves /HPF
400
300
200
100
0
control
trained
non-trained
D
NF200
(afferent)
pos. nerves /HPF
110
100
90
80
70
60
50
control
trained
non-trained
E
NPY
pos. nerves /HPF
325
300
275
250
225
200
175
150
control
trained
non-trained
F

Table 1

Urodynamic parameters

Group	non-voiding contractions (NVC)	maximal bladder capacity (MBC) [ml]	leak point pressure (LPP) [cmH_2O]
Intact	-	0.55±0.07	17.13±1.34
Trained	2.7±11	1.78±0.47	10.11±1.3
Non-trained	12.6±5.2	3.45±0.39	14.63±2.36
p-Value	0.037	0.004	0.454

Table 2

Renal function and bladder morphology

Group	Serum creatinin [µmol/l]	Cystatin C [mll/l]	Bladder wall thickness [µm]	Co:SM ratio
Intact	30.20±0.91	<0.05	432±16	0.405±0.04
Trained	31.29±1.58	<0.05	486±35	0.350±0.03
Non-trained	29.33±1.33	<0.05	677±50	0.220±0.02
p-Value	1.0	-	0.004	0.009

Co:SM ratio: collagen to smooth muscle ratio

Table 3

Immunolabeling of specific nerve fibers in the bladder wall

Group	PGP9.5 general nerves/HPF	NF200 afferent A-fibers/HPF	NF200:PGP9.5 A-fiber fraction	NPY efferent fibers/HPF	TH adrenergic fibers/HPF	VAChT cholinergic fibers/HPF
Intact	203.3±18.0	93.7±4.8	0.465±0.02	233.0±1.9	274.2±30.2	267.03±32.1
Trained	304.8±16.0	88.7±6.1	0.232±0.02	254.9±15.5	268.09±24.3	196.33±19.5
Non-trained	110.2±3.1	58.6±3.4	0.537±0.04	164.4±6.9	247.84±10.4	197.05±24.3
p-Value	<0.001	0.006	0.001	<0.001	0.552	0.985

PGP9.5 = protein gene product 9.5, HPF = high power field. NF200 = neuro filament 200, NPY = neuropeptide Y, TH = thyroxin hydroxilase, VAChT = veicular acethylcholine transporter